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(54) Title: MOLECULAR DETECTION OF CHROMOSOME ABERRATIONS

(57) Abstract

The invention relates to the field of cytogenetics and the application of genetic diagnostic techniques in pathology and haematology. Specifically, the invention relates to nucleic acid probes that can be used in hybridisation techniques for the detection of chromosomal aberrations and other gene rearrangements such as immunoglobulin (Ig) and T cell receptor (TCR) gene rearrangements. The probes provided by the invention are a distinct and balanced pair of probes of comparable size each preferably being from 1 to 100 kb, or smaller, and flanking a potential breakpoint in a chromosome.

Title: Molecular detection of chromosome aberrations.

The invention relates to the field of cytogenetics
and the application of genetic diagnostic techniques in
pathology and haematology. Specifically, the invention
relates to nucleic acid probes that can be used in
5   hybridisation techniques for the detection of chromosomal
aberrations and other gene rearrangements such as
immunoglobulin (Ig) and T cell receptor (TCR) gene
rearrangements.

Chromosomal aberrations are a leading cause of
10   genetic disorders or diseases, including congenital disorders
and acquired diseases such as malignancies. At the basis of
the above malignancies lies the fact that all cells of a
malignancy have a common clonal origin. Chromosomal
aberrations in malignancies stem from rearrangements,
15   translocations, inversions, insertions, deletions and other
mutations of chromosomes, but also losses or gains of whole
chromosomes are found in malignancies. In many chromosome
aberrations two different chromosomes are involved. In this
way, genes, or fragments of genes are removed from the normal
20   physiological context of a particular chromosome and are
located to a recipient chromosome, adjacent to non-related
genes or fragments of genes (often oncogenes or proto-
oncogenes). Such an aberrant genetic combination can be the
foundation of a malignancy.
25   Often, such rearrangements involving two non-aberrant
chromosomes happen in a somewhat established pattern. Breaks
occur in either of the two chromosomes at a potential
breakpoint or breakpoint cluster region resulting in removal
of a gene or gene fragment from one chromosome and subsequent
30   translocation to the other chromosome thereby forming a
rearranged chromosome where the rearranged fragments are
fused in a fusion region.

Detection of chromosome aberrations can be achieved using a wide array of techniques, various of which entail modern biomolecular technology. Traditional techniques such as cytogenetic analyses by conventional chromosome banding
5   techniques are, although highly precise, very labour intensive, require skilled personal and are expensive. Automated karyotyping is useful for some diagnostic applications, such as prenatal diagnosis, but is ineffective in analysing the complex chromosomal aberrations of many
10   malignancies. Furthermore, above techniques require fresh (cultured) cells, which are not always available.

Other, more modern, techniques are Southern blotting or other nucleic acid hybridisation techniques or amplification techniques such as PCR, for the detection of
15   well-defined chromosome aberrations for which suitable nucleic acid probes or primers are available. With these techniques, fresh or frozen cells can be used, and sometimes even samples after formalin fixation as long as the nucleic acid sequences to be hybridised or amplified remain intact
20   and accessible. However, even with above modern technology, several disadvantages can be found that hamper the application of these diagnostic techniques in the rapid screening for chromosomal aberrations related to said malignancies.

25   Southern blotting lasts 3 to 4 weeks, which is too slow for efficient diagnosis and choice of therapy in malignancies, and allows only 10-15 kb of nucleic acid sequences to be analysed per probe analysis.

PCR, although in essence well-suited for rapid and
30   massive diagnostic testing or even screening, allows only 0.1 to 2 kb of nucleic acid to be analysed per PCR analysis, which greatly hampers rapid screening of vast stretches of chromosomes and breakpoint cluster regions within the chromosomes. An additional disadvantage of PCR is its

inherent sensibility to mismatched primers. Small, normal, and physiological alterations which can always be present in the nucleic acid sequence of the gene fragment complementary to the primer hamper the reliable application of PCR and
5   eventually give rise to false-negative results. Especially false-negative results render a PCR-based diagnostic test, albeit very specific, not sensitive enough for reliable diagnosis, and it goes without saying that only a reliable diagnosis of malignancies can contribute to an understanding
10 of the prognosis and the design of an adequate therapy.

       Fluorescent *in situ* hybridisation techniques (FISH) are less dependent on the complete matching of nucleic acid sequences to get positive diagnostic results. In general FISH employs probe analyses with large, mainly unspecified,
15 nucleic acid probes that hybridise, however often with varying stringency, with the genes or gene fragments located at both sides of the fusion region in the rearranged chromosome in the malignant cell. Using large probes renders the FISH technique very sensitive. The binding of the
20 colocalizing probes is generally detected either directly or indirectly with fluorochromes and visualised via fluorescence microscopy of a population of cells obtained from the sample to be tested.

       However, even the currently used FISH protocols have
25 inherent disadvantages, these mainly relate to the selection of nucleic acid probes employed in the current FISH protocols, which can give false-positive results in the diagnosis of chromosomal aberrations. For example, probes directed against different chromosomes with juxta position of
30 signals in case of translocation create a rather large risk of false-positive results. Hence, the diagnostic tests are, albeit sensitive, not specific enough to employ standard FISH techniques in massive or rapid diagnostic testing, let alone in automated testing or screening.

Thus far, generally large probes, derived of cosmid clones, YAC clones, or other cloned DNA fragments have been used as probes in FISH. The exact position of these probes in relation to the fusion region in the rearranged chromosome is

5   unknown and they are of largely unspecified and varying genomic length (genomic length or distance as expressed as the number of nucleotides or bases (b)) and go without specific selection or modification of these probes beyond the mere labeling of the probes with the necessary reporter

10   molecules i.e. fluorochromes. For designing or selecting probes, little or none guidance is given in the art, beyond mere suggestions as to where to localise putative probe. False-positive results obtained with these probes may stem from aspecific hybridisation with a wide array of (major)

15   repetitive sequences present throughout various chromosomes, or from cross-hybridisation to homologous sequences in the genome, or from overlap of the used probes with the breakpoint cluster region or from the difference in signal intensities as far as originating from size differences of

20   the probes. These causes of false-positive results are frequently not recognized. False-positive results are especially detrimental to rapid diagnosis if rapid or routine screening of patients is needed to detect malignancies or in evaluating treatment protocols. A false-positive result then

25   necessitates cumbersome re-testing of patients, or even unsuspecting clients that have been submitted to routine screening protocols, and can greatly alarm these people. Furthermore, translocations are generally detected with two different probes, one for each of the involved chromosomes,

30   which probes than colocalise during the *in situ* hybridisation in case of a translocation, but show separate signals when no translocation is present (see for example EP 0430402, EP 0500290 A, Tkachuk et al., Science 250, 559-562, 1990; Tkachuk et al., Clinical applications of fluorescence in situ

hybridization", Genetic analysis techniques and applications
vol. 8, 67-74, 1991). However, in practice 2 to 4% of normal
interphase cells tested by FISH will show false-positive
results due to the fact that the two probes colocalize by
5   chance. An additional disadvantage of the current FISH
protocols is that it is in practice necessary to know both
chromosomes that are involved in the translocation as well as
the relevant breakpoint regions of both chromosomes to define
the nucleic acid probes enabling the detection of the
10   specified translocation, while as yet unknown or ill-defined
translocations originating from a well-known gene and an
unknown partner gene remain undetected.

        The present invention provides nucleic acid probes
that can be used in diagnostic testing for chromosome
15   aberrations which combine a high sensitivity and a high
specificity. The probes provided by the invention can
hybridise, *in situ* or *in vivo* or *in vitro* with complementary
nucleic acid molecules such as (m)RNA or DNA, as e.g.
transcribed by or found in (non-aberrant and/or rearranged)
20   chromosomes. The present invention provides for each
translocation analysis a distinct and balanced pair of
nucleic acid probes. The probes are distinct in that they
each hybridise to a different sequence specifically selected
and flanking a distinct potential breakpoint in a non-
25   aberrant chromosome. Furthermore, the pair formed by e.g.
probe A and probe B is distinct from the pair formed by e.g.
probe A and probe X. Furthermore, in above example probes A,
B and X constitute three pairs, A-B, B-X and A-X. The probes
in said pair are comparable or balanced in that they are
30   designed to be of for example comparable size or genomic
length with the final aim of facilitating the generation of
signals of comparable intensity. In addition, said probes can
be comparably labelled with reporter molecules resulting in
signals of comparable intensity. In addition, said probes can

each be labelled with a different fluorochrome, facilitating
detection on one spot of different colour when they
colocalize when no aberration is detected. Also, said probes
can be selected to react with a chromosome, at respective

5   complementary hybridisation sites that are located at
comparable distances at each side of a breakpoint or
breakpoint cluster region of a chromosome. The distinct and
balanced pair of nucleic acid probes provided by the
invention entails probes that are for example of comparable

10 size or genomic length, each probe of the pair for example
being from 1 to 10 kb, or 7 to 15 kb, or 10 to 20 kb, or 15
to 30 kb, or 20 to 40 kb, or 30 to 50 kb, or 40 to 60 kb, or
50 to 70 kb, or 60 to 80 kb, or 70 to 90 kb, or 80 to 100 kb
in length. By using such a distinct and balanced pair of

15 probes flanking a breakpoint region and not overlapping the
corresponding fusion region, false-positive diagnosis in
hybridisation studies is avoided. The invention further
provides a distinct and balanced pair of nucleic acid probes,
each being labelled with at least one different reporter

20 molecule. Nucleic acid probes can be labelled with
chromophores or fluorochromes (e.g. FITC or TRITC) or by
introducing a hapten such as biotin and digoxigenin.
Fluorochrome labelled probes can be detected directly.
Hybridisation with haptenised nucleic acid probes is followed

25 by indirect detection using chromopores, fluorochromes or
enzymes such as peroxidase. The invention further provides a
distinct and balanced pair of nucleic acid probes which are
characterised in that both probes hybridise to a single
corresponding nucleic acid molecule or its complementary

30 strand, or hybridise to one (non-aberrant) chromosome, or
hybridise to a fragment thereof, possibly comprising the
aberration, instead of two probes that hybridise separately
to the two chromosomes that are involved in a given
translocation, as currently used in hematology and oncology

in general (see for example Tkachuk et al., Science 250, 559-562, 1990; Tkachuk et al., Clinical applications of fluorescence in situ hybridization", Genetic analysis techniques and applications vol. 8, 67-74, 1991).

5   The invention further provides a distinct and balanced pair of nucleic acid probes which hybridise to said nucleic acid molecule at a genomic distance of no more than 100 kb, but preferably no more than 50 kb. In addition the invention provides a distinct and balanced pair of nucleic

10   acid probes which hybridise in situ, and can i.e. be used in diagnostic tests entailing FISH techniques. Furthermore, the invention provides a distinct and balanced pair of nucleic acid probes which probes each hybridise in situ under varying but generally low-stringent conditions to only a few DNA

15   molecules per cell. The nucleic acid probes composed of several DNA fragments are tested either on metaphase spreads or with Southern blotting for hybridisation sensitivity and specificity to select the probe on containing as little major repetitive sequences as possible, to avoid high background

20   staining. The nucleic acid probes are tested in fiber FISH (i.e. hybridisation on extended single DNA fibers immobilised on glass slides), prior to being employed in diagnostic testing, for mapping and checking their relative positions.

      The probes are tested for example to avoid using

25   probes hybridising two repetitive sequences. Probes can consist of sets of various oligonucleotides, thereby avoiding repetitive sequences present in a flanking region. Such sets are distinctly labelled, with separate or distinct reporter molecules for each probe (or set of oligonucleotides) that is

30   aimed at the respective flanking region. Such probes can each consist of multiple labelled oligonucleotides, each hybridising to a distinct area in a flanking region. One probe can for example contain from 10 up to 200 of such oligonucleotides, preferably from 50-150, each

oligonucleotide for example being 10-20 nucleotides ling. For example, the intron-exon structure of the *MLL* gene is described in the Br J Haematol 1996;93:966-972. The manuscript also shows that most breakpoints in the *MLL* gene

5   are located between exon 9 and exon 14. PNA containing probes can be designed in exon 3 to 8 for the "upstream FISH probe" and in exon 15 to 31 for the "downstream FISH probe". Particularly exon 4 and exon 28 are important for probe design, because these two exons are rather large and

10   therefore can contain most of the PNA probes. PNA oligonucleotides can be synthesized for example for their capacity to hybridise with said exon 4 or exon 28 from the 119Q3 target gene and used in one cocktail as probe for one flanking region.

15   The invention further provides the use of said distinct and balanced pair of probes in diagnostic testing for chromosome aberrations. The pair of probes according to the invention can be used in the detection of nucleic acid comprising the aberration or fragments of the aberration, or

20   in the detection of cells, *in situ* or *in vitro*, comprising the chromosome aberration. The invention thus provides a pair or pairs of distinct and balanced probes which can be used in the detection of disorders or diseases related to chromosomal aberrations, i.e. malignancies, such as haematopoietic

25   malignancies as further explained below. Furthermore, the invention provides a diagnostic kit or assay comprising a pair of nucleic acid probes according to the invention which can be used in the detection of disorders or diseases related to chromosomal aberrations, i.e. malignancies, such as

30   haematopoietic malignancies with such a diagnostic kit or assay provided by the invention it is e.g. possible to monitor the effects of therapy and detect minimal residual disease or detect early relapse of cancer. One can also identify the origin of bone marrow cells following bone

marrow transplantation. One can also detect viral sequences, and their localisation in the chromosome, in cells. More in detail the present invention is described while referring to molecular detection of chromosome aberrations in

5   hematopoietic malignancies but is widely applicable for analysis of chromosome aberrations in general.

The development of reliable probes for detection of well-defined or even ill-defined chromosome aberrations in hematological malignancies is described as non-limiting

10   example to illustrate the invention. Such probes can be used for diagnosis and for molecular classification of the involved malignancies. The new probes can be used in diagnostic testing in several types of hematological malignancies with increased sensitivity, specificity, and

15   efficacy of analysis.

Each year world-wide many cases of hematopoietic malignancies are being diagnosed. In the European Union (~375 million inhabitants) this concerns ~98,000 patients per year. The estimated number of patients in the USA (~250 million

20   inhabitants) is ~65,500 per year. The majority of hematological malignancies are of lymphoid origin: acute lymphoblastic leukemias (ALL), chronic lymphocytic leukemias, most malignant lymphomas, and multiple myelomas. The non-Hodgkin's lymphomas (NHL) form the largest group,

25   representing approximately half of all hematopoietic malignancies. Furthermore, European epidemiological studies show that the incidence of NHL is gradually increasing (~5% per year), which indicates that NHL poses a significant public health problem in Europe and most probably throughout

30   the Western world. Although the annual number of patients diagnosed with ALL is smaller than for NHL, ALL has a high prevalence in children, representing the most frequent malignancy in childhood.

Lymphoid malignancies consist of a broad range of ~25 different disease entities, which differ in clinical presentation, prognosis, and treatment protocols. These disease entities have been defined in the recent Revised

5 European American Lymphoid neoplasm (REAL) classification. In this classification the lymphoid malignancies are divided in B-cell malignancies (~90%) and T-cell malignancies (~10%).

The diagnosis and classification of lymphoid malignancies is generally based on cytomorphology and

10 histomorphology, complemented with immunophenotypic information via flow cytometry and/or immunohistochemistry. This immunophenotypic information appears to be valuable for classification of lymphoid malignancies, such as the classification of ALL into pro-B-ALL, common-ALL, pre-B-ALL,

15 and several types of T-ALL. In mature B-cell malignancies with immunoglobulin (Ig) expression the diagnosis can be supported by immunophenotypic clonality assessment via detection of single Ig light chain expression, i.e. the distribution of Igκ and Igλ positive B-cells, which is

20 heavily skewed in case of a B-cell malignancy.

The value of clonality assessment is based on the fact that all cells of a malignancy have a common clonal origin. In lymphoid malignancies this is reflected by the presence of identically (clonally) rearranged Ig and T cell

25 receptor (TCR) genes: clonal Ig and/or TCR gene rearrangements are found in most (90-95%) immature lymphoid malignancies and virtually all (>98%) mature lymphoid malignancies. Therefore molecular clonality analysis of Ig and TCR genes is highly suitable for discrimination between

30 monoclonal (malignant) and polyclonal (reactive) lymphoproliferations. Suspect lymphoproliferations should therefore be subjected to molecular clonality assessment.

During the last decade the knowledge about genetic aberrations in hematopoietic malignancies has considerably

increased, especially in acute leukemias and NHL. Currently, well-established chromosome aberrations are found in 35-40% of ALL and in 30-40% of NHL. These chromosome aberrations can be used as alternative or additional markers for molecular

5   clonality assessment. More importantly, these chromosome aberrations appear to be relevant classification markers, which supplement the currently used morphological and immunophenotypic classification systems. It has been clearly demonstrated that several genetic aberrations are associated

10   with a favourable prognosis, whereas others are associated with poor prognosis, such as t(4;11) in pro-B-ALL and t(9;22) in common-ALL. Several treatment protocols have started to use this information for stratification of treatment. Therefore it can be anticipated that rapid and reliable

15   detection of well-defined genetic aberrations will become essential in the diagnosis and management of hematopoietic malignancies.

Several different types of chromosome aberrations have been identified in ALL and NHL. The chromosome

20   aberrations in precursor-B-ALL mainly concern translocations, which result in fusion genes, encoding for fusion proteins with new or modified functions. Examples include the E2A-PBX and BCR-ABL fusion proteins, resulting from t(1;19) and t(9;22), respectively. Another important chromosome region,

25   the 11q23 region with the MLL gene, is involved in several types of translocations in acute leukemias. In these 11q23 translocations different partner genes are involved, leading to different fusion proteins. One of them is t(4;11), which is observed in ~70% of infant acute leukemias. Many

30   chromosome aberrations in T-ALL and NHL involve Ig or TCR gene sequences in combination with oncogene sequences. These chromosome aberrations do not give rise to fusion proteins, but result in increased or stabilized expression of the involved oncogene, thereby contributing to uncontrolled

growth. They occur at relatively high frequency in particular disease categories, such as t(14;18) with involvement of the *BCL2* gene in ~90% of follicular lymphomas and t(11;14) with involvement of the *BCL1*/Cyclin D1 gene in ~70% of mantle cell

5   lymphomas.

From origin cytogenetic analysis of chromosomes has been the standard technique for detection of chromosome aberrations. This technique needs the presence of cells in metaphase, which generally requires various cell culture

10   systems, dependent on the type of malignancy. The success rate for obtaining reliable karyograms is highly dependent on the type of malignancy and the experience of the laboratory and ranges from less than 50% to over 90%. Furthermore, some chromosome aberrations can not or hardly be detected by

15   cytogenetic analysis such as *TAL1* deletions in T-ALL and t(12;21) in precursor-B-ALL. Therefore in case of well-established chromosome aberrations the labor-intensive and time-consuming classical cytogenetics is now being replaced by molecular techniques. As said, molecular analysis of

20   genetic aberrations can be performed with Southern blotting, polymerase chain reaction (PCR) techniques, and FISH techniques.

Southern blot analysis has long been the most reliable molecular method for detection of well-established

25   chromosome aberrations, but this technique is dependent on the availability of suitable DNA probes, which recognize all relevant breakpoint cluster regions of the involved chromosome aberrations. The latter probably explains why *BCL2* and *BCL1*/CyclinD1 gene aberrations are detectable by Southern

30   blotting in only 75% of follicular lymphomas and in only 50% of mantle cell lymphomas, respectively. Furthermore, Southern blot analysis is time-consuming and requires relatively large amounts of high-quality DNA derived from fresh or frozen cell samples.

Over the last five years, PCR-based techniques have
been developed as alternatives for Southern blotting. PCR
techniques have the advantage that they are rapid and require
minimal amounts of medium-quality DNA, which might even be
5   obtained from formalin-fixed paraffin-embedded tissue
samples.   Also mRNA can be used after reverse transcription
(RT) into cDNA. RT-PCR is especially valuable in case of
chromosome aberrations with fusion genes and fusion
transcripts, such as frequently seen in precursor-B-ALL and
10   in t(2;5) in anaplastic large cell lymphoma. Despite these
obvious advantages, the broad application of PCR techniques
for detection of chromosome aberrations in hematopoietic
malignancies is hampered by several problems. False-negative
PCR results can be obtained if the DNA or mRNA from formalin-
15   fixed paraffin-embedded tissue samples is less optimal than
anticipated, or when primers are mismatching. False-positive
results might be obtained due to cross-contamination of PCR
products between samples from different patients; especially
in case of RT-PCR studies of fusion gene transcripts it might
20   be difficult to exclude false-positive results. Finally,
routine PCR analysis can only be used to study relatively
small fusion regions of chromosome breakpoints (<2 kb). This
implies that multiple oligonucleotide primer sets are needed
to cover the most important breakpoint and fusion regions,
25   whereas it will be difficult to study large breakpoint or
fusion regions (>10 kb). This explains the lower
detectability of chromosome aberrations, and thus again the
presence of false-negative results, at the DNA level by PCR
as compared to Southern blotting.
30   A major advantage of FISH techniques as compared to
cytogenetic analysis, Southern blotting, and PCR analysis is
that FISH can be performed on interphase nuclei of all kind
of tissue and cell samples and that there is no need for
extraction of DNA or mRNA. In FISH techniques generally large

DNA probes (>25 kb) are used, which are located around the breakpoint regions of the two chromosomes of the studied chromosome aberration. This implies that FISH probes can scan much larger regions than Southern blot probes or PCR primers.

5 This advantage is especially important for detection of breakpoints outside the traditional breakpoint cluster regions. Furthermore the use of large fluorescently-labeled DNA probes allow direct and rapid visualization of deletions and translocations of the studied gene regions. Application

10 of the latest generation of fluorescent microscopes with multiple fluorochrome filter combinations, CCD camera, and appropriate computer software allow the combined use of multiple FISH probes, which are labeled with different fluorochromes.

15 The availability of suitable probes is the main limiting factor in using FISH technology for detection of chromosome aberrations. Thus far, generally cosmid clones, YAC clones, or other cloned DNA fragments have been used without specific selection or modification of these probes.

20 For many of these probes the position in the genome is not precisely known; they often even overlap with breakpoint cluster regions, and they often contain repetitive sequences which cause high background staining. Furthermore, translocations are generally detected by use of two different

25 probes, one for each of the involved chromosomes; these two probes are assumed to colocalize in case of a translocation, but show separate signals if no translocation is present. However, in practice 2 to 4% of normal interphase cells will show false-positive results due to the fact that the two

30 signals colocalize by chance.

For routine applicability of FISH techniques or other probe analysis assays or kits for the detection of chromosome aberrations in the diagnosis and classification of

hematopoietic malignancies, it is necessary to design distinct and balanced probes.

1. The probes of the invention are selected to form a distinct and balanced pair of nucleic acid probes;

5 size of the probes is each within certain limits of the genomes to be detected (e.g. 1-10, or 10-30, or 20-40, or 30-50, or 40-60 kb), with the final aim that the intensity of the fluorescent signals of the various probes is comparable.

2. In an additional embodiment of the invention

10 the position of the probes constituting the pair is determined precisely, i.e. no overlap with breakpoint cluster regions, the relevant breakpoints are preferably located within 50 kb or preferably even within 25 kb of either probe, and an additional probe pair has to be designed, if two

15 breakpoint regions of a particular chromosome aberration are separated for more than 30-50 kb depending on the exact position of the probes.

3. In a further embodiment the nucleic acid probes do not contain (major) repetitive sequences, and do

20 not cross-hybridise, which results in high background staining. For this reason the nucleic acid probes composed of several fragments can be tested either on metaphase spreads or with Southern blotting for hybridisation sensitivity and specificity.

25 4. The nucleic acid probes can alternatively, or additionally be tested in fiber FISH prior to being employed in diagnostic testing, for mapping and checking their relative positions.

5.   It has additionally been found that detection
of chromosome breakpoints becomes easier and more reliable,
if two separate probes, labelled with two different
fluorochromes, constituting said pair are designed around one
5 of the breakpoint regions of a chromosome aberration. This
will lead to colocalization of the signals if no breakpoint
is present. However if a breakpoint occurs in the studied
breakpoint region, the two differently labeled probes will
result in two separate signals.

10   6.   In addition, the design of a third probe
(labeled with a third fluorochrome) and thus the design of
two additional distinct pairs of probes for the partner gene
of the chromosome aberration allows precise identification of
the chromosome aberration.

15

Chromosome aberrations found in malignancies are
useful for molecular classification, such as in case of acute
leukemias, malignant lymphomas and solid tumors (Table 1).
However, several of these aberrations are more important than
20 others, because of their high frequency or because of their
prognostic value. For instance, t(14;18) occurs frequently in
NHL, whereas t(12;21) is frequently found in childhood
precursor-B-ALL. On the other hand, translocations involving
the MLL gene in the 11q23 region represent a poor prognostic
25 factor and the presence of 11q23 (MLL gene) aberrations is
already in use as an important factor for stratification of
treatment in acute leukemias. Also t(9;22) in ALL has a poor
prognosis and is used for treatment stratification
The MLL (for myeloid-lymphoid leukemia or mixed-
30 lineage leukemia) gene in chromosome region 11q23 is involved
in several translocations in both ALL and acute myeloid
leukemias (AML). In these translocations the MLL gene,
encoding a protein that shows homology to the Drosophila
trithorax gene product, is fused to partner genes on

different chromosomes. To date at least ten partner genes
have been identified. Some of these translocations, like the
t(4;11)(q21;q23), t(11;19) (q23;p13) and t(1;11)(p32;q23),
predominantly occur in ALL, whereas others, like

5 t(1;11)(q21;q23), t(2;11)(p21;q23), t(6;11)(q27;q23) and
t(9;11)(p22;q23) are more often observed in AML. Other types
have been reported in ALL as well as AML. Treatment-induced
AML with 11q23 aberrations can arise in patients previously
treated with topoisomerase II inhibitors. Rearrangements

10 involving the 11q23 region occur very frequently in infant
acute leukemias (around 60-70%), and to a much lesser extent
in childhood and adult leukemias (each around 5%). *MLL* gene
rearrangements, especially the t(4;11), have been shown to be
a poor prognostic factor in infant leukemias, resulting in a

15 3-year overall survival of 5% as compared to 85-90% in cases
with germline *MLL* genes.

The large *MLL* gene (>100 kb) consists of 21 exons,
encoding over 3900 amino acids. Breakpoints in the *MLL* gene
are clustered in a 8.5-9 kb region that encompasses exons 5-

20 11. Because of its relatively small size, this breakpoint
region is easily accessible for molecular detection of
translocations. By choosing two distinctly-labeled FISH
probes in the sequences flanking the breakpoint region, any
translocation involving the 11q23 region can be detected on

25 the basis of segregation of the two fluorochrome signals,
whereas the two fluorochromes colocalize when no
rearrangement in the *MLL* gene has occurred. Furthermore, the
use of a third fluorochrome for probes directed against
partner genes enables the identification of the precise type

30 of translocation. This two-step approach of FISH analysis
guarantees efficient and direct detection of all aberrations
involving the 11q23 (*MLL* gene) region in the first step,
whereas in the second step the type of 11q23 translocation
can be determined.

18

Chromosome aberrations in lymphoid malignancies often involve Ig or TCR genes. Examples include the three types of translocations (t(8;14), t(2;8), and t(8;22)) that are found in Burkitt's lymphomas, in which the *MYC* gene is coupled to

5   Ig heavy chain (*IGH*), Ig kappa (*IGK*), or Ig lambda (*IGL*) gene segments, respectively. Another common type of translocation in this category is the t(14;18)(q32;q21) that is observed in ~90% of follicular lymphomas, one of the major NHL types. In this translocation the *BCL2* gene is rearranged to regions

10   within the *IGH* locus within or adjacent to the JH gene segments. The result of this chromosome aberration is the overexpression of the BCL2 protein, which plays a role as survival factor in growth control by inhibiting programmed cell death.

15   The *BCL2* gene consists of only three exons, but these are scattered over a large area. Of these the last exon encodes a large 3' untranslated region (3' UTR). This 3' UTR is one of the two regions in which many of the t(14;18) breakpoints are clustered and is called "major breakpoint

20   region" (mbr); the other breakpoint region involved in t(14;18) translocations, is located 20-30 kb downstream of the *BCL2* locus and is called the "minor cluster region" (mcr). A third *BCL2* breakpoint area, the vcr (variant cluster region), is located at the 5' side of the *BCL2* locus and is

25   amongst others involved in variant translocations, i.e. t(2;18) and t(18;22), in which *IGK* and *IGL* gene segments are the partner genes.

By choosing a set of FISH probes that are located in the regions upstream of the mbr region and downstream of the

30   mcr region, translocations in these regions can be detected upon segregation of the fluorochrome signals. An additional set of FISH probes is designed for the vcr region, since the distance between the vcr region and the other two breakpoint clusters is far too large (~400 kb) to use the same probes.

As a second step in all these approaches, FISH probes in the
IGH, *IGK*, and *IGL* genes are used for identification of the
exact type of translocation.

Several types of nucleic acid probes can be employed
5   in FISH technology as provided by the invention for detection
of chromosome aberrations. Each of these probe types has its
own characteristic features and advantages, together
constituting a complementary approach, i.e. cosmid, PAC, or
YAC derived probes, PCR-based probes, or PNA-based probes.

10   Clones obtained from cosmid, PAC or YAC libraries
constitute large probes that, when labeled with
fluorochromes, result in appropriate signals upon
hybridization (Gingrich et al., 1996). However,
conventionally as the precise position of these probes is
15 unknown, there often is a risk of overlap with the breakpoint
cluster region of the involved chromosome aberration, if no
further selection or modification of these probes is
performed. Furthermore, such large probes often contain
repetitive sequences, which cause high background staining.
20 Distinct and balanced pairs of probes comprising cosmid, PAC
or YAC probes that are designed to react with the flanking
regions upstream and downstream of the breakpoint area on one
of the involved chromosomes can therefore be exactly
positioned in fiber FISH experiments or by use of Southern
25 blotting using small well-defined inclusion and exclusion
probes which are designed around the breakpoint area avoiding
said overlap with the breakpoint cluster. The presence of
potential repetitive sequences is excluded via Southern blot
analysis of genomic DNA.

30   Probes that are generated by PCR have the additional
advantage that they can be positioned exactly, however for
this approach sequence information is required at least in
the areas for designing the target-specific PCR primers for
producing the probes. Once generated the PCR products are

checked for the presence of repetitive or cross-hybridizing sequences that hamper specific detection of the flanking regions upstream and downstream of the involved break point cluster. PNA-based probes for FISH technology comprise
5   multiple (e.g. 50-150) distinct PNA oligonucleotides each of which shows a typical size of 5-40, more typically 10-25 nucleotides and which together produce an appropriate signal for detection of chromosome aberrations using FISH technology. PNA probes, having a neutral peptide backbone to
10   which the four deoxynucleotides are coupled are stable nucleic acid fragments that hybridize to complementary nucleic acid sequences with high affinity (Egholm et al., 1993; Corey, 1997). Due to the fact that mismatches strongly influence PNA hybridization, sequence specificity of PNA
15   recognition can be easily achieved, therby rendering PNA probes highly selective probes to be used in FISH technology. PNA probes have now been used in a variety of applications inclusing *in situ* hybridization to highly repetitive contromeric or telomeric sequences (Corey, 1997). Thusfar,
20   only a single PNA oligonucleotide directed against repeated sequences was sufficient for appropriate signal intensities. The design of a balance pair of nucleic acid probes comprising multiple (e.g. 50-150) distinct PNA oligonucleotides directed against target sequences in the
25   flanking regions of a breakpoint cluster provides detection of chromosome aberrations as well as with other nucleic acid probes.

Applicability of the various types of probes
30

Each of the before mentioned types of FISH probes has its specific applicability, but together they constitute complementary and partly overlapping strategies.

The *MLL* gene in chromosome region 11q23 is an example of a detectable region that is involved in several translocations in both ALL and AML (Table 1), providing a perfect example of a chromosome aberration for which PCR-

5   based or PNA-based FISH probes can preferably be designed. Because the breakpoint area in the *MLL* gene is so tightly clustered with ample exons available in the flanking regions upstream and downstream of the breakpoint cluster, sequence-based design and production of distinct and balanced pairs of

10 PCR-based and/or PNA-based FISH probes is very useful in this chromosome region. Design of precisely positioned cosmid or PAC clones could be useful as an alternative or additional strategy.

The *BCL2* gene area is an example that is involved in

15 several chromosome aberrations in malignant lymphomas (Table 1) which contains several breakpoint areas which are located outside the coding sequence at the 5' and 3' sides of the *BCL2* locus and which are lying far apart. The *BCL2* locus therefore exemplifies a gene involved in chromosome

20 aberrations for which distinct and balanced pairs of FISH probes are more difficult to generate via PCR and/or via pooling of PNA oligonucleotides, as less sequence information is available. In such chromosome aberrations distinct and balanced pairs of cosmid, PAC, and/or YAC derived FISH probes

25 can be employed after careful selection and modification of the exact position.

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Table 1. Examples of chromosome aberrations in malignancies, that are detectable with a distinct and balanced pair of nucleic acid probes of the invention.

Translocation	Involved genes	Primary target gene for FISH probe design	Occurrence per disease category
Acute leukemias			
t(4;11) (q21;Q23)	*MLL-AF4*		- 70% of infant ALL
t(11;19)(q23;p13)	*MLL-ENL*	*MLL* gene	- 5-7% of ALL
t(6;11)(q27;q23)	*MLL-AF6*		- 5-6% of AML
t(9;11)(p22;q23)	*MLL-AF9*		
Malignant lymphomas			
t(14;18)(q23;q21)	*BCL2-IGH*		- 90% of follicular NHL
t(2;18)(p12;q21)	*IGK-BCL2*	*BCL2* gene	- 25% of immunoblastic NHL
t(18;22)(q21;q11)	*IGL-BCL2*		- 25% of diffuse large cell centroblastic NHL
			- 5-10% of B-CLL
Solid tumors			
t(11;22)(q24;q12)	*EWS-FLI1*		>95% of Ewing sarcoma
t(21;22)(q22;q12)	*EWS-ERG*	*EWS* gene	
t(7;22)(p22;q12)	*EWS-ETV1*		

Claims

1.   A pair of nucleic acid probes of comparable size, each
preferably being from 1 to 100 kb, more preferably each being
from 1 to 10 kb, or 7 to 15 kb, or 10 to 20 kb, or 10 to 30
kb, or 20 to 40 kb, or 30 to 50 kb, or 40 to 60 kb, or 50 to
70 kb, or 60 to 80 kb, or 70 to 90 kb, or 80 to 100 kb, and
flanking a potential breakpoint in a chromosome.

2.   A pair of nucleic acid probes according to claim 1,
each being labeled directly or indirectly with at least one
reporter molecule.

3.   A pair of nucleic acid probes according to claim 2
wherein the reporter molecule is selected from the group
consisting of enzymes, chromophores, fluorochromes, haptens
(such as biotin or digoxygenin).

4.   A pair of nucleic acid probes according to any of
claims 1 to 3 characterised in that probes hybridise to a
single corresponding nucleic acid molecule.

5.   A pair of nucleic acid probes according to claim 4
wherein the corresponding nucleic acid molecule is at least a
fragment of a chromosome.

6.   A pair of nucleic acid probes according to claim 5
wherein the chromosome is not aberrant.

7.   A pair of nucleic acid probes according to any of
claims 4 to 6 which hybridise to said nucleic acid molecule
at a genomic distance of no more than 100 kb, but preferably
no more than 50 kb.

8.   A pair of nucleic acid probes according to any of
claims 1 to 7 which hybridise *in situ*.

9.   A pair of nucleic acid probes according to any of the
claims above which probes each hybridise *in situ* under low-
stringent conditions to only a few linear DNA molecules per
cell.

10.   Use of a pair of nucleic acid probes according to any of claims 1 to 9 for the detection of a nucleic acid molecule comprising a chromosome aberration.

11.   Use of a pair of nucleic acid probes according to any of claims 1 to 9 for the detection of cells comprising a chromosome aberration.

12.   Use of a pair of nucleic acid probes according to any of claims 1 to 9 for the detection of a disorder or disease caused by a chromosome aberration.

13.   Use of a pair of nucleic acid probes according to any of claims 10 to 12 wherein the chromosome aberration is related to a malignancy.

14.   Use of a pair of nucleic acid probes according to any of claims 10 to 12 wherein the chromosome aberration is related to a hematopoietic malignancy.

15.   A diagnostic kit comprising at least a pair of nucleic acid probes according to any of claims 1 to 9.

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12Q1/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6   C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TKACHUK ET AL.: "Detection of bcr-abl fusion in chronic myelogeneous leukemia by in situ hybridization" SCIENCE, vol. 250, 1990, pages 559-562, XP002044140 see the whole document	1-15
X	TKACHUK D C ET AL: "CLINICAL APPLICATIONS OF FLUORESCENCE IN SITU HYBRIDIZATION" GENETIC ANALYSIS TECHNIQUES AND APPLICATIONS, vol. 8, no. 2, 1 April 1991, pages 67-74, XP000262526 see the whole document	1-15
X	EP 0 430 402 A (UNIV CALIFORNIA) 5 June 1991 see the whole document	1-15

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[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

* Special categories of cited documents :

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"P" document published prior to the international filing date but later than the priority date claimed

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INTERNATIONAL SEARCH REPORT

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate. of the relevant passages	Relevant to claim No.
X	EP 0 500 290 A (UNIV CALIFORNIA) 26 August 1992 see the whole document ---	1-15
A	WO 96 17958 A (UNIV CALIFORNIA ;MEDICAL RES COUNCIL (GB)) 13 June 1996 see the whole document ---	1-15
A	THOMPSON C T ET AL: "CYTOGENETIC PROFILING USING FLUORESCENCE IN SITU HYBRIDIZATION (FISH) AND COMPARATIVE GENOMIC HYBRIDIZATION (CGH)" JOURNAL OF CELLULAR BIOCHEMISTRY. SUPPLEMENT, no. SUPPL. 17G, 1993, pages 139-143, XP000612761 see the whole document ---	1-15
A	US 5 538 869 A (SICILIANO MICHAEL J ET AL) 23 July 1996 see the whole document ---	1-15
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